Population Genetics of Vascular Epiphytes; Optimization of DNA Extraction and Cross Species Transference in *Tillandsia usneoides*

A Thesis submitted
To the Graduate School
Valdosta State University

in partial fulfillment of requirements
for the degree of

MASTER OF SCIENCE

in Biology

in the Department of Biology
of the College of Science & Mathematics

October 2020

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ABSTRACT

Vascular epiphytes are found in more than 70 different families and make up 9-10% of the 290,000 described species of vascular plants. Despite their significant contribution to vascular flora diversity, they have received far less attention in population genetic studies than their terrestrial counterparts. This study had three goals: (I) Provide a comprehensive literature review on the population genetics of vascular epiphytes to answer questions such as, which taxa have been surveyed and where did these studies take place? What types of molecular markers have been used? What types of population genetic measures have been reported? What are the general findings across different taxa? And what journals have published these sorts of studies? (II) Develop a DNA extraction technique for an ecologically and culturally important epiphytic plant, *Tillandsia usneoides* (Spanish moss) that produces high yields of DNA while also minimizing possible contaminants that could affect downstream processes. (III) Asses the utility of cross-species transference as a method to develop usable microsatellite DNA primers for *T. usneoides*. In the literature review, I found 25 published studies in 13 journals that studied population genetics of vascular epiphytes, representing three taxonomic families in seven countries and four continents. To date, seven types of molecular markers have been used. Interestingly, these markers do not follow typical usage trends that has been observed in other groups, with allozymes being used more frequently than microsatellite DNA, and with no published studies to date that have used SNPs. Taxonomic and geographic biases in the population genetic literature reflect general patterns observed in the ecological literature, with most studies conducted in North America and focused on Orchidaceae (41%), Polypodiaceae (36%) and Bromeliaceae (23%). Due to the diversity of life history strategies observed in epiphytes,

it is difficult to generalize population genetic results across taxa; nevertheless, in ferns, there was evidence of high gene diversity and genotype frequencies that conformed to Hardy-Weinberg expectations, whereas results were more variable for orchids and bromeliads. It is difficult to generalize results across the different taxa studied, this is believed to be reflect the diversity of life history strategies observed in epiphytes. For the DNA extraction study, we found that a modified CTAB extraction produced the highest yield and highest quality DNA. Utilizing extracted DNA from *T. usneoides,* cross-species transference of microsatellite primers from seven different taxa in the family Bromeliaceae was assessed. Of the 36 primer sets that were screened, we identified 15 loci that produced fragments that were close in size to what was reported in the primer notes, but only 7 of the 15 loci produced polymorphic loci without other apparent abnormalities. For the seven loci, the number of alleles ranged from 2 to 12. Although limited in number, the primer sets that we identified should be sufficient for assessing broad scale population genetic patterns in *T. usneoides*.

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ACKNOWLEDGMENTS

I cannot begin to express the gratitude I feel for the people who have helped get me this far. Thank you to my committee chair, Dr. Corey Anderson for taking me on as one of his graduate students and guiding me through my time here. Also, to my committee members, Dr. Cristina Calestani, Dr. Eric Chambers and Dr. Russell Goddard. Your advice was invaluable, and I certainly would not have made it this far without it. A special thanks to Dr. Brian Ring, who although was not on my committee, helped me more times than I can count.

To all my friends, getting to know the other graduate students was certainly the highlight of this program. Having you all around made the time spent here far easier than if done alone. Special recognition to Blake Lamb, Christopher Le and Damion Castellano who helped with sample collection. I would also like to thank Danielle Ward; I could not have asked for a better lab mate. Her friendship and presence in the lab made this process much easier.

Finally, I wish to thank my family whose words of encouragement and support has kept me going during my time here. I am beyond grateful.

Chapter 1

BACKGROUND

Epiphytes are “air plants” that germinate and root non-parasitically on other plants at all stages of life (Zotz, 2016). Although a proper count on the total number of epiphytes is not available, there are approximately 28,000 described vascular epiphytes. One of the most notable taxonomic families with considerable epiphyte diversity, is Bromeliaceae. Bromeliaceae is a large family of flowering plants found mostly in the neotropics and with the exception of Orchidaceae, has the largest number of known epiphytic organisms (Givnish et al., 2014). Their recent adaptive radiation is thought to be the reason for their considerable morphological and ecological diversity (Zanella et al., 2012). Despite this diversity, there is relatively little variation in terms of ploidy levels, with most studied organisms being 2n = 50 (Gitai et al., 2005). Genetic diversity of Bromeliaceae has only been lightly studied (Zanella et al., 2012).

Perhaps one of the most morphologically recognizable Bromeliads is *Tillandsia usneoides* (Spanish moss). *Tillandsia usneoides* is a culturally important and ecologically significant epiphytic bromeliad with a geographic range that extends from South America to the southeastern United States. There have been a number of ecological studies of this species (Angelini & Briggs, 2015; Barve et al., 2014; Billings, 1904; Garth, 1964; Penfound & Deiler 1947), but, like other species of Bromeliaceae, genetic studies are lacking.

Tillandsia usneoides grows in warm and humid climates and its distribution is thought to be constrained by climatic factors that affect flowering phenology (Garth, 1964; Barve et al., 2014; Barve et al., 2015). It is more frequently found on *Celtis* spp. and *Quercus virginiana* and less frequently on *Juniperus* and *Liquidambar* while also being uncommon on other species (Callaway et al., 2002; Barve et al., 2014). *Tillandsia usneoides* has a scorpioid dichotomous growth pattern (Garth, 1964) and forms long hanging festoons along the branches of its host tree, but it can also be found on abiotic structures such as power lines and fences. It has scaly trichomes that aid in water absorption and retention (Billings, 1904). Little is known genetically about Spanish moss other than the fact that it has 16 short chromosomes. *Tillandsia usneoides* is an ecologically important species because, when present in high abundance on a host tree, it provides a refuge for other species (especially juvenile insects), resulting in a facilitation cascade that ultimately augments species richness both on the tree and in the leaf litter (Angelini & Briggs, 2015; Thomsen et al., 2018). Nevertheless, many questions about Spanish moss dispersal, including the relative frequency of different reproductive modes (i.e., seed dispersal vs. clonal dispersal by vegetative breakage) have not been resolved (Garth, 1964). This large geographical range of *T. usneoides* has been attributed anecdotally to the vegetative mode of reproduction, although again, little to no genetic work has been done to support this.

Chapter 2

POPULATION GENETICS OF VASCULAR EPIPHYTES

Introduction

Population genetics is a field of study that has grown in significance over the past century. It involves studying genetic variation within populations and modeling changes in the frequency of alleles over space and time. One of the most important aspects of population genetics in plants, and perhaps one of the most difficult, is trying to quantify dispersal: whether it be of seeds or pollen (Ouborg et al., 1999). Studying genetic diversity in epiphytes reveals a lot about their life history strategies. Having genetic data available allows us to understand different mating systems, detect seed and pollen dispersion and determine how species undergo colonization (González-Astorga et al., 2004; Kartzinel et al., 2013; Trapnell et al., 2013). How these questions get answered depends largely on what type of molecular marker is employed in the study. For example, allozymes have been used throughout the literature to estimate gene flow. However, allozymes have largely become obsolete over time due to the development of DNA-based markers such as microsatellites and single nucleotide polymorphisms (SNPs). Along with molecular makers, the type of population genetic measures also depends on the question being asked. If a study had a phylogeographic aspect, then they might use DNA sequence data (e.g. from chloroplast DNA). This may come with its own set of genetic measures

that are different from genotype-based measures, such as private haplotypes (HP) and haplotype diversity (HD).

Epiphytes (air plants) are plants that germinate and root non-parasitically on other plants at all stages of life. The epiphytic life form is found in more than 70 different families, comprising 9 to 10% (~28,000) of all vascular plants (Zotz, 2016). Most epiphytes are members of three families, Orchidaceae, Bromeliaceae and Polypodiaceae. Orchidaceae contains 68% of all known epiphytes (~19,000) while Bromeliaceae contains approximately 1,800 species. Although a specific count is not available, the family Polypodiaceae also has many epiphytes, with 74 genera containing epiphytes and representing 50% of all epiphytic ferns. Despite their significant contribution to vascular flora diversity, population genetic data on vascular epiphytes is scarce (Bush et al., 1999; Cascante-Marin et al., 2014; Cortés-Palomec et al., 2019; González-Astorga et al., 2004; Hooper & Haufler, 1997; Solórzano et al., 2010; Soltis et al., 1987). Even though there have been relatively few population genetic studies of epiphytes, other aspects of epiphyte biology have been studied intensively. For example, Zotz (2016) did a bibliometric analysis of ecological studies in which he found 2753 different articles. This shows that the lack of attention may not be on the organism itself, but in the field of population genetics.

In this study we examined the peer-reviewed population genetic literature on vascular epiphytes to quantify methodological trends and findings, addressing questions such as: (1) Are there any trends in which taxa are being examined and where these studies are geographically taking place; (2) What types of molecular markers have been used on epiphytes and how have patterns of usage changed over time?; (3) Which

population genetic measures have been used; (4) What are the general findings across different taxa; and (5) in which journals have population genetic studies of epiphytes been published.

Materials and methods

Definition of an epiphyte

To summarize the population genetic literature on epiphytes, an acceptable definition of the term "epiphyte" must first be addressed (Zotz, 2016). Some plant species that do not strictly exhibit the stereotypic characteristics one would associate with a "traditional" epiphyte, but still exhibit epiphytic tendencies. For example, while mistletoes do grow and live on other plants, the one caveat that prevents them from being "true epiphytes" is the fact that they are parasitic. Other areas of confusion stem from plant species that can grow not only epiphytically, but also terrestrially and as lithophytes. There are other categories of epiphytes that have been proposed in hopes of clearing up some of these grey areas when discussing this group of organisms; for example, there are obligate epiphytes, accidental epiphytes, and facultative epiphytes. Each having to do with whether the majority of the species grows epiphytically, terrestrially or within some range of the two, respectively. For the purposes of this review, we will use the definition provided by Zotz (2016) in which he defines epiphytes as plants that germinate and root non-parasitically on other plants at all stages of life.

Literature search

An extensive literature search on population genetics of vascular epiphytes was conducted using “Google scholar”, “Galileo”, “NCBI” and “Web of science” with various combinations of the following keywords: “epiphyte”, “diversity”, “population”, “genetic”, and “variation”, “genetic structure”, “Bromeliaceae”, “Orchidaceae” and “Polypodiaceae”. Once this method was exhausted, we “reversed searched” the already obtained articles to see what articles cited those authors. Baseline criteria were established for articles to be considered in this review. First, the main focus of the published article was on vascular epiphytes and, second, the study utilized at least one type of molecular marker for genotyping (e.g. Allozymes, RFLPs, or microsatellites). Articles were reviewed and information was placed into nine categories: author, year of publication, journal, number of species, taxon, country, continent, molecular markers, and population-genetic coefficients.

Results

Geographic and taxonomic trends

A total of 25 published articles on epiphyte population genetics were identified, spanning seven countries and four continents (Fig.1 & 2). Most studies have been conducted in Costa Rica and Mexico; together they make up 55% of published works on the population genetics of epiphytes (Table 1). Second is the United States, representing 19% of the studies and Brazil was third with 15%. The remaining 11% of the studies were conducted in Ecuador, Madagascar, and South Korea (each representing 3.7% of published works). There were only two studies that spanned multiple countries: Hooper

and Haufler (1997) surveyed for isozyme variability in six species of Polypodiaceae in Mexico and Costa Rica and Kartzinel et al. (2016) sampled populations of *Catopis nutans* along the Pacific slope of Costa Rica and in Florida (US).

Approximately 70% of studies focused on a single species, 17% studied two species and 13% studied three or more species, with the greatest number of species being studied by Hooper and Haufler (1997), who examined a total of six species in the family Polypodiaceae. In most cases, if a study examined multiple species, all species were from the same taxonomic family. However, Bush et al. (1999) included species from both Orchidaceae and Polypodiaceae.

Even though the epiphytic life form represents 912 genera in 73 families (Zotz, 2016), population genetic studies have only examined three of these families to date. Of the known studies, 41% have been conducted on Orchidaceae, 36% on Polypodiaceae, and 23% on Bromeliaceae (Table 1).

Usage of molecular markers over time

Over the 35-year span between the first and last of the 25 published studies, seven types of molecular markers were used (Fig. 3). Allozymes /isozymes were used most frequently (= 42.5%), followed by microsatellites (21.5%). Other markers included AFLPs (11%), chloroplast DNA (cpDNA) markers (11%), RAPDs (7%), cpDNA haplotypes (3.5%) and plastid sequences (3.5%).

Population genetic measures and findings

In the 25 published studies, population genetic measures were reported 145 times. The most commonly reported measure (31%) was Wright's *F*-statistics (i.e. F_{ST}, F_{IS}, or

F_{IT}). In this class of population genetic measure, the most frequently used estimator was the variance-based measures of Weir and Cockerham's (1984), used 63% of the time. The next most frequent was Nei's (1977) estimators based on heterozygosity, which were used 19% of the time. The other 18% were estimators that were only used once such as Li and Horvitz (1953), a Fragment frequency approach (which is a non-allelic method of estimating F_{ST} that assumes fixed homozygosity at each locus) and one based on Slatkin's R_{ST} (1995) estimator. Many studies (17%) also provided information about polymorphism by providing the number of alleles or private alleles per locus; proportion of polymorphic loci (P) was reported 10% of the time (Fig. 4). Observed heterozygosity (H_O) and expected heterozygosity (H_E) were reported 11.7% and 10.3% of the time. The remaining 20% of the data consist of coefficients that were seldom used in other studies (Table 2).

A little more than half of the studies (52%) used analysis of molecular variance (AMOVA) to assess genetic diversity at different hierarchical levels. Interestingly, even though a large portion of studies utilized AMOVA, only three of them assessed fine-scale genetic structure of their study species (Cortes-Palomec et al., 2019; Trapnell et al., 2004; Torres et al., 2018). Other studies that did not use AMOVA sought to answer questions simply about genetic variation to provide insight for conservation efforts. Only five studies compared pollen and seed dispersion in some way (Kartzinel et al., 2013; Palma-Silva et al., 2009; Trapnell et al., 2004; Trapnell et al., 2005; Trapnell et al., 2013). Clustering algorithms have become popular in the field of population genetics since around the year 2000 when microsatellite DNA became prevalent. However, it was not until Palma-Silva et al. (2009) that this clustering method was used. Since then, only five

studies have reported the use of a Bayesian clustering analysis. Three studies used it to make some inference about population genetic structure (Goetze et al., 2018; Soares et al., 2018; Torres et al., 2018). One study done by Winkler et al. (2011) used this approach to compliment a Jaccard distance-based analysis to compare the population genetic structure of two ferns that differ in their ability to colonize secondary habitats. Each study used STRUCTURE software to run clustering analysis based on admixture models.

Journal type

There were 13 journals that have published literature on population genetics of epiphytes. With 5 out of the 25 articles, Molecular Ecology published the most papers on epiphyte population genetics, followed by American Journal of Botany, Heredity, and Selbyana with three published papers each. Annals of Botany and Biotropica each had two published papers, and there were seven journals that had one published study (Table 3).

Discussion

Surprisingly, a limited number of population genetic studies have been conducted on vascular epiphytes. We have found that only the families Orchidaceae, Bromeliaceae and Polypodiaceae have been studied with Costa Rica and Mexico being the main regions where population genetic studies of epiphytes have been conducted. The types of molecular markers used in these studies still seem to rely on older techniques such allozymes/isozymes, with newer markers such as microsatellites only being used early as 2009. More recently developed markers, such as single nucleotide polymorphisms

(SNPs) have not been used at all. Although there were some population genetic measures that were commonly used among studies, such as Wright's *F*-statistics and expected and observed heterozygosity, there was still considerable variation in the types of measures used, which most likely reflects variation in the types of molecular markers used, the types of questions asked, and the different approaches each study took in learning about their respective study organisms.

Epiphytes have been asserted to be an ideal model to study population genetic structure because of their colonization habits, patchy ecological and geographical distribution patterns and specific pollination strategies (González-Astorga et al., 2004). Despite this, they have received relatively little attention by plant population geneticists. Ávila-Díaz and Oyama (2007) proposed that even though epiphytes are diverse and ecologically important, they receive less attention because of difficulties in accessing the canopy. Not only does there seem to be a physical barrier to obtaining samples of vascular epiphytes, but there may also be a political one as well. Most researchers cannot easily travel to another country for sample collection. Mori et al. (2012) suggest that obtaining the necessary permits to travel to tropical countries and collect specimens for molecular studies can take years and are not always successful.

We have found that there is both a taxonomic and geographic bias in the literature of population genetics of vascular epiphytes. Interestingly, Zotz (2016) found similar taxonomic and geographic trends in surveying ecological studies in vascular epiphytes which outnumber population genetic studies 100 fold. It was found the Orchidaceae, Bromeliaceae and "ferns" had received far more attention than other families. He also noted that most ecological studies were from the Americas and attributes this to the high

epiphytic richness of the Neotropics. This coincides with our findings that only the same three families have been studied and the majority of studies take place in neotropical regions such as Mexico and Costa Rica. This makes it clear that it is not just in the field of population genetics that there are biases but in vascular epiphytes overall.

When compared to the other studied families of vascular epiphytes, Orchidaceae has received the most attention. Orchids, in general, are prized as ornamentals by plant fanciers, which would give them an intrinsic value that would make them interesting to study. There have been numerous population genetic studies of Orchidaceae, but most of these studies are heavily biased towards terrestrial species despite the fact 69% of all orchids are epiphytic (Alcantara et al., 2006; Ávila-Díaz & Oyama, 2007; Bush & Kutz, 2006). Furthermore, Orchids also comprise 68% of all epiphytes so they may serve as an organism of convenience (Zotz, 2016). It is also suggested that there is interest in studying orchids for their relative contributions of pollen and seed dispersal because of how far seeds have the potential to move while pollen flow is limited by pollinator movement (Trapnell & Hamrick, 2004).

The studies that have been conducted on epiphytes in the family Orchidaceae have tended to try and resolve more complex questions about seed dispersion, pollen movement and colonization (Kartzinel et al., 2013; Trapnell & Hamrick, 2004; Trapnell & Hamrick, 2005; Trapnell et al., 2013). While most studies have examined patterns of genetic variation over large geographic extents, Trapnell et al. (2004), Torres et al. (2018) and Cortés-Palomec et al. (2019) assessed fine-scale genetic structure at the level of the forest and tree. Results of population genetic studies of orchids vary from study to study.

Some showed mean expected heterozygosity near zero and high levels of inbreeding, while others showed high genetic diversity and little deviation in genotype frequencies from Hardy-Weinberg expectations (Alcantara et al., 2006; Ávila - Díaz & Oyama 2007; Chung et al., 2007).

Epiphytic pteridophytes were underrepresented in the population genetic literature. There are seven families that contain 30 or more species of epiphytes, but there have only been population genetic studies on species within the family Polypodiaceae (Hooper & Haufler, 1997; Ranker, 1992). Although Polypodiaceae has 74 genera that contain epiphytes and make up about 50% of all epiphytic ferns, the families Aspleniaceae and Dryopteridaceae are the two genera that contain the most species, with more than 400 each (Zotz, 2016). Even though there are only four studies done on the family Polypodiaceae, in total 13 species have been examined. Interestingly, three of these studies (which account for 92% of the studied fern species) found evidence of high genetic diversity and genotype frequencies that conformed to Hardy-Weinberg random mating expectations, implying that ferns may generally exhibit high levels of out-crossing (Hooper & Haufler, 1997; Ranker, 1992; Winkler et al., 2011). Unlike the families Orchidaceae and Bromeliaceae, which each had considerable variation in terms of the reported results.

In epiphytic bromeliads, there are species that show high genetic diversity because they are self-incompatible and are considered obligate out-crossers (Goetze et al. 2018). While other bromeliad species such as *Vriesea reitzii* have mixed systems of outcrossing and clonality (Soares et al. 2018), resulting in considerable variability in levels of genetic diversity, depending on the dominant reproductive mode in an area.

Conversely, some epiphytic bromeliads have been shown to have very low variation. For example, Soltis et al. (1987) used allozymes to examine genetic diversity in *Tillandsia recurvata* within an arid region of northern Mexico and found that *T. recurvata* exhibited fixed homozygosity at all loci, supporting autogamy. Solórzano et al. (2010) used microsatellite loci on *T. recurvata* collected from several tree species in the semi-arid region of central Mexico and found that levels of variation were low, but higher than that reported by Soltis et al. (1987), suggesting that this species is not strictly autogamous (Cascante-Marin et al., 2014). Low genetic diversity and autogamous mating systems have been reported to be not uncommon among bromeliads (Cascante-Marin et al., 2014; Lavor et al., 2014; Zanella et al., 2011)

This review has found that molecular marker usage in epiphytes does not necessarily follow the stereotypical temporal trends. It is expected that the temporal pattern would be as follows: isozymes/allozymes, AFLPs (amplified fragment length polymorphism) and microsatellites, then SNPs (single nucleotide polymorphism). However, we found that isozymes/allozymes were the most popular marker type for population genetic studies of epiphytes and have been used as recently as 2013. As expected, microsatellites and AFLPs became more popular in the same 10-year span, starting around the year 2000, but did not necessarily exceed allozyme studies. While SNPs have been used with increasing frequency through time, there has not been the widescale shift towards SNPs in the last decade that has been seen in other taxa.

The importance of coefficients in population genetics cannot be overstated, as they are what allow researchers to measure and compare genetic variation within and among populations. *F*-statistics and allelic measures are commonly used in population

genetics and it is not necessarily surprising that they were frequently reported in population genetic studies of epiphytes. The measures used that fall in the "other" category include private haplotypes (H_p), haplotype diversity (H_D) which would be used to assess variation in chloroplast DNA sequences.

It is difficult to generalize the findings about genetic diversity and patterns among study systems for all publications because of the variety of questions asked, the approach taken to answer those questions, how the data were presented and what results were reported. In the family Polypodiaceae, basic summary statistics such as mean number of alleles per locus (A) and expected heterozygosity (H_E) were readily reported. Findings reported in studies within Bromeliaceae were not as homogenous as the findings for Polypodiaceae. In the family Bromeliaceae for example, there was variation even when papers examined the same species. As previously mentioned, Soltis et al. (1987) and Solórzano et al. (2010) both studied *T. recurvata* with the former coming to the conclusion that it showed zero genetic variability and was completely autogamous, while the latter found that this wasn't the case and attributed this to the fact that Soltis et al. (1987) used allozymes, which are less variable than microsatellite markers. Other studies dealing with Bromeliaceae varied in the reported results and the conclusions they drew. In the five papers that used microsatellites (Cascante-Marin et al., 2014; Goetze et al., 2018; Palma-Silva et al., 2009; Soares et al., 2018; Solórzano et al., 2010), average H_E = 0.62 was much higher than the two papers (González-Astorga et al., 2004; Soltis et al., 1987) that used allozymes, average H_E = 0.09. These studies generally had genotype frequencies that deviated from Hardy-Weinberg expectations. Data reported by population genetic studies done on epiphytic orchids show a similar pattern to that of the

bromeliads in the fact that it is hard to compare the different studies because of the variety of mating systems, the inconsistencies with how data was presented, and the variety of methods used across the different studies. For example, Zanella et al. (2012) found that markers with relatively high polymorphism such as microsatellite DNA gave vastly different values than older markers such as allozymes. Reported polymorphism in orchids is relatively low, with an average of 2.1 alleles per locus. Deviations from Hardy-Weinberg equilibrium were observed across multiple studies.

Out of the 12 journals that have published articles on the population genetics of epiphytes, nearly half were published in *Molecular Ecology,* followed by *Heredity* and *Conservation Genetics*. This large number of publications in *Molecular Ecology* is not surprising considering that this is the main outlet for empirical population genetic research, and it has a higher current impact factor (IF) than the rest of the journals (Table 3).

Conclusion

Based on the results of this review, there has been a lack of work on the population genetics of epiphytes. This is made evident by the fact that between the first found published paper by Soltis et al. (1987) and the most recent paper by Cortés-Palomec et al. (2019), there have been only 25 published articles. It can also be said that there is a bias for certain taxa specifically in the study of population genetics of vascular epiphytes. Of the 73 families that are home to at least one epiphytic organism, only species in the three families Orchidaceae, Polypodiaceae and Bromeliaceae have been studied. Additional population genetics studies of epiphytes would help to clarify many

basic aspects of their natural history that are not well understood and may be critically important in developing effective conservation management strategies for critically endangered species. While there have been no published studies of epiphytes to date that have utilized genomic approaches to marker development (such as RADseq), such methods might be most promising and cost efficient for epiphytes because thousands of codominant genotypes could be screened to identify polymorphic loci that could be used to examine genetic structure at multiple hierarchical levels. If canopy access and travel continue to limit research, the growth of data on population genetics of vascular epiphytes will remain stagnant.

Chapter 3

OPTIMIZATION OF DNA EXTRACTION IN *TILLANDSIA USNEOIDES*

Introduction

When conducting genetic work, it is important to have a DNA extraction protocol that produces sufficient yields of high-quality DNA. It has been reported that varying levels of polysaccharides, polyphenols, and secondary metabolites make it difficult to obtain high-quality DNA from some species of plant (Aboul-Maaty & Oraby, 2019; Inglis et al., 2018; Sahu et al., 2012). These contaminants could affect downstream processes such as PCR and cloning, giving erroneous results. If the concentration of extracted DNA is not sufficient then that would require the use of a higher volume of sample, making genomic samples a limiting factor. Commercially available kits use silica based spin columns to obtain high quality DNA, but the yields are, in some cases, low. Homemade DNA extraction protocols have been proven to be an effective and cost-efficient way to extract sufficient amounts of high-quality DNA (Aboul-Maaty & Oraby, 2019; Allen et al., 2006; Jobes et al., 1995; Porebski et al., 1997). Some of the most common methods use CTAB (cetyl trimethylammonium bromide), a cationic detergent that aids in the lysis of cells. Commercially available extraction kits have been optimized to work on a broad range of different study systems. However, the exact contents of their kits are usually proprietary so that makes it difficult to adjust concentrations to better suit individual

studies. Although homemade protocols may be more time-consuming than commercial kits. you have more control over exactly what goes into the extraction process and can optimize for a particular species.

The problem of insufficient yields and low-quality DNA when using commercial kits may be exacerbated in plants that have reduced leaf structures and tissues, as found in certain species of epiphytes. For example, species in the genus *Tillandsia* (family Bromeliaceae) have reduced leaf structures and stem axis that is covered with specialized trichomes that help them to absorb moisture and nutrients from the atmosphere. For species, such as *Tillandsia usneoides* (Spanish moss), most of the plant volume is comprised of lignified cells (sclerenchyma) that serve as support structures. While these adaptations make well adapted to absorbing moisture and nutrients from the atmosphere, there is minimal leafy tissues that are ideal for DNA extraction.

In a preliminary study, we used two extraction kits, Qiagen and Viogene to extract DNA from *T. usneoides*, however, yields were relatively low and often contained secondary metabolites. This is not uncommon when working with plants that contain a lot of polyphenols and polysaccharides (Inglis et al., 2018). Here we developed a modified cetyl trimethylammonium bromide (CTAB) extraction to increase yields and quality of DNA and compared our modified CTAB extraction to results from a commercially available DNA extraction kit (Viogene Plant Genomic DNA Extraction Miniprep System).

Materials and methods

CTAB and Viogene DNA extraction methods comparison

Twenty-four samples (strands) of Spanish moss were all obtained from the same festoon. Two extraction protocols were compared to determine which would provide a higher DNA yield. We used a modified Viogene kit extraction protocol on one half of the samples, and the CTAB extraction on the other half. The Viogene kit uses a silica gel spin column to bind DNA and has a relatively short extraction time. The new growth was submerged into liquid nitrogen, pulverized using mortar and pestle, ground into a fine powder and then transferred into 1.5 ml Eppendorf tubes (~ 700 µl of tissue relative to the total volume of the tube). The first step of each protocol required 400 µl of lysis buffer and 4 µl of RNase to be added to the ground sample prior to an incubation step of 65°C for 10 minutes. For our purposes, better yields were obtained when the amount of buffer and RNase was doubled to 800 µl and 8 µl respectively while also extending the incubation time to 18–24 hours. From here the protocol was followed according to the manufacture's procedure. To test if there was a difference in the mean DNA yields for the Viogene extraction method and the homemade CTAB method, we conducted paired *t*-test for unequal variances using R software version 4.0.2 (R Core Team, 2020).

CTAB extraction

For the modified Cetyl TrimethylAmmonium Bromide (CTAB) extraction, if collected samples were not used immediately, they were stored at -20°C until extraction could begin. Approximately 0.1 g of "new growth" from a single strand of Spanish moss was ground into a fine powder. Ground samples were placed in microcentrifuge tubes and kept in a closed container of liquid nitrogen until ready for the full extraction process.

One microliter of 10^5 µg/ml RNase per sample was added to pre-warmed CTAB buffer containing 0.2% 2-Mercaptoethanol (extraction buffer). Five hundred microliters of extraction buffer was added to each sample and then incubated at 65°C overnight. To remove tissue debris, the lysate was centrifuged at 12,000 rpm at 24 °C for 3–5 minutes. Once the lysate had been isolated, one volume of a 1:1 mixture of phenol and chloroform was added. The sample was then centrifuged again at 12,000 rpm at 24 °C for 5–10 minutes and the aqueous layer was removed; one volume of chloroform was mixed thoroughly into the solution to clean up any residual phenol. The sample was then centrifuged again at 12,000 rpm at 24 °C for 5–10 minutes, and the aqueous layer was again removed, placed in a clean tube and one volume of cold isopropanol was then added to precipitate the DNA (at -20°C overnight). After precipitation, the samples were then centrifuged at 12,000 rpm at 24 °C for 10–15 minutes. The isopropanol was then discarded and the remaining pellet was washed twice with 500 µl of 70% ethanol, and then air dried to remove all traces of ethanol. One hundred microliters of pre-warmed (37°C) TE buffer was then added to each sample and samples were then incubated for at least one hour at 37°C. DNA samples were quantified using a Qubit® 2.0 Fluorometer with a dsDNA BR (broad range) Assay kit. In order to assess the purity of the extracted DNA, a NanoDrop™ spectrophotometer was used for subsequent DNA quantification and purity analysis.

Results

CTAB and Viogene comparison

Although it had a much higher variance, the average DNA yields for the CTAB extraction (mean = 74.4 ng/µl; sd = ± 31.0) was greater than the mean yields obtained from the Viogene kit (mean = 7.2 ng/µl; sd = ± 4.4) (Fig 5). The paired *t*-test showed a significant difference in yield amounts between methods (t= -7.4, df = 11, $p < 0.001$). The average 260/280 ratio for the CTAB method was 1.77, while the average for the Viogene kit was 2.9.

Discussion

DNA extraction trials revealed that the CTAB extraction method consistently produced higher yields (~10x) of DNA when compared to the Viogene kit. This is perhaps why it is common place in plant DNA extractions because it is good at minimizing the contaminants commonly associated with plant genomic extractions such as polysaccharides, polyphenols, secondary metabolites, and RNA (Aboul-Maaty & Oraby, 2019; Jobes et al., 1995; Wang et al., 2012). A few downsides to this method are, that it can be time consuming and some steps need to be completed under a fume hood because it requires harsh chemicals such as phenol and chloroform. The Viogene kit on the other hand has a much quicker protocol and employs the use of silica gel spin columns that would normally make sure only high-quality DNA gets eluted in the final steps. This is not the case when dealing with *T. usneoides* as indicated with the average yields and 260/280 ratios.

In the early stages of the study, fragment analysis (Chapter 4) resulted in electropherograms that were atypical. This resulted in the question of whether the peak

patterns obtained were the result of poor-quality DNA or multiple genetic profiles in a single sample. After trying a few different extraction protocols, we are confident that the modified CTAB protocol resolves any concerns about the purity of the DNA samples. The 260/280 ratios of the tested samples had an average of 1.77, with a ratio of 1.8 being considered optimal. This new protocol also resulted in high yields of DNA, much higher than the yields obtained from the Viogene extraction kit (Fig. 5). Viogene was chosen as the kit for comparison because it was found that it outperformed Qiagen by having on average higher DNA yields (Brown & Griffin, 2016).

Although acceptable 260/280 ratios were obtained, spectra produced by the NanoDrop™ spectrophotometer indicated that samples may still have phenol contamination. This was resolved by modifying the extraction protocol to combine phenol and chloroform in a 1:1 ratio and having an extra chloroform treatment to remove any residual phenol (Fig. 6). This resulted in spectra profiles typical of a good quality DNA sample. Nevertheless, potential phenol contamination did not seem to have an effect on downstream fragment analyses: after comparing electropherograms before and after these modifications the major peaks themselves (the scored peaks) remained unchanged.

Here we have provided a reliable CTAB protocol that not only produces a high yield of genomic product but also minimizes possible contaminants. This DNA extraction protocol provides a foundation for further genetic and genomic studies of *T. usneoides*, as well as studies of anatomically similar species in the genus *Tillandsia*.

Chapter 4

CROSS SPECIES TRANSFERENCE OF MICROSATELLITE PRIMERS FOR GENETIC MARKER IDENTIFICATION IN SPANISH MOSS (*TILLANDSIA USNEOIDES*)

Introduction

There are numerous genetic markers available to plant geneticists. In a broad sense, these markers can be classified into two groups: classical and DNA/molecular (Nadeem et al., 2017). Older biochemical techniques such as isozymes/allozymes fall in the classical group. They are different from DNA-based markers in that they are variants of enzymes based on gene products. Even though they have become relatively obsolete, they still remain a popular marker in some situations. This is perhaps because they are cost effective and have long been studied leading to well-developed protocols that do not require de novo development. Markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) are examples of DNA/molecular markers. Unlike biochemical techniques, these markers are based on DNA rather than gene products meaning they are more informative markers. Perhaps most popular in the DNA/molecular group are simple sequence repeats (SSRs, commonly referred to as "microsatellites") and single nucleotide polymorphism (SNPs). Microsatellites consists of repeat motifs (e.g. CA) that

are distributed throughout the genome of eukaryotes. Their high mutation rates and co-dominant inheritance make them ideal for assessing genetic variation in populations. Even though markers such as SNPs have exponentially increased in popularity since the late 1990s, microsatellites still have several advantages over SNPs such as generally higher allelic richness and less issues with missing data and reproducibility of loci across populations (Guichoux et al., 2011).

One drawback of microsatellites is the fact that in some cases they must be developed de novo for a species (Merritt et al., 2015), a process that can be time-consuming and expensive. This sometimes forces researchers to rely on cross species transference of primer sets developed in other closely related species. If the primer sequences are conserved, there may be successful transferability even if species are not closely related (Louise dos Santos et al., 2007). The success of cross species transference has been well documented, especially in bromeliads with seven different studies utilizing the technique.

In this study, we examined the utility of cross species transference to develop polymorphic microsatellite DNA primer sets for *Tillandsia usneoides*, by using 15 different primer sets originally characterized for nine different genera of the family Bromeliaceae (Table 4). Some of these primer sets have been commonly used in genetic studies of bromeliads with some of them being used to study more than seven different species of bromeliads. The primer sets come from eight different taxa listed in (Table 4). *Tillandsia usneoides* is an ecologically and culturally important epiphyte, yet, to date, no population genetic studies have been conducted on this species. Because there have been no genetic markers developed for *T. usneoides,* in this current study we hope to identify

viable markers for future use in population genetic research on *T. usneoides* to better understand the frequency of its different modes of reproduction and patterns of gene flow. Because of the way *T. usneoides* grows and is potentially dispersed, we also assess if there are multiple individuals mixed in a single festoon.

Materials and methods

Preliminary work

First, we screened whether the chosen primer sets produced visible fragments. Samples of *T. usneoides* were first collected from host trees around Valdosta State University and from various locations in Lowndes County, Georgia (30.8600° N, 83.2934° W). Samples were collected and immediately sifted and new growth was separated from older tissue until enough material was gained to cover the bottom of a petri dish. Initially, DNA was extracted using two commercial plant DNA extraction kits, DNeasy Plant Mini kit (Qiagen, Hilden, Germany) and Plant Genomic DNA Extraction Miniprep System (Viogene, Taipei, Taiwan), following modified protocols (Chapter 3).

Primer sequences were obtained from "primer notes" that were published for other species in the family Bromeliaceae. We used cross-species transference tables from these publications to identify primer sets that had produced a visible fragment for *T. usneoides* or that consistently showed successful amplification across different genera (Table 6). Thirty-six microsatellite DNA primer sets were screened, six of the primers were amplified in *T. usneoides* and 15 were amplified in species of the same genus *Tillandsia* (Table 7). PCR reaction (50 µl) were prepared with 25 µl of Promega GoTaq G2 Hot Start Colorless Master Mix (Promega Corp., Madison, WI, USA), 5 µl of 10 µM forward and reverse primers, 2 µl of genomic template and 13 µl of ddH_2O. The thermal cycling conditions consisted of a denaturation phase of 95°C for 90 seconds, an annealing

phase of 51°C for 30 seconds and final extension phase 72°C for 30 seconds; this was repeated a total of 35 cycles. The PCR products were then size fractionated on a 1.8% agarose gel stained with ethidium bromide. The gels were imaged with an ImageQuant™ LAS 4000 (GE Healthcare, Chicago, USA). Fragment size was calculated using exACTGene 50bp mini DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) as a reference. If there was a visible band (indicating successful amplification), loci were further screened with an Agilent 2200 TapeStation (Agilent Technologies, Waldbronn, Germany) to confirm approximate fragment size (polymorphism could not be adequately assessed). This TapeStation requires one microliter of PCR product to be mixed and vortexed for 5 seconds with 10 µl of genomic DNA sample buffer. The combination of agarose gel and TapeStation screening allowed us to identify 26 out of 36 primer sets that produced a visible fragment; of those 26, 15 were chosen for further trials because their observed size ranges were similar to the expected size ranges reported in the published literature.

Sample collection for current study and mixed profile analysis

Samples of *T. usneoides* used in this study were collected from 16 different locations (Table 5). If samples were not used immediately, they were stored in individual collection bags and kept at -20°C until processing. The number of samples collected from each site varied depending on the coverage of *T. usneoides* in the area and accessibility of the canopies of host trees. Samples were gathered from what was considered distinct festoons (i.e. not touching another festoon) on the same and surrounding host trees. This was often difficult in areas where the density of *T. usneoides* was high because when there is considerable coverage, one cannot always delineate discrete festoons. Because

study resources were limited and it is was not clear how many genets were contained on a host tree (or in a forest stand), we decided to sample over a wide geographic extent (multiple counties and several political states), rather than subsample within the same area, as the our goal was to identify polymorphic loci for further analysis, rather than to estimate population genetic parameters within subpopulations. The majority of the samples used (~85%) were gathered around Lowndes County, GA, USA. There were three samples gathered from Beaufort County, SC, USA and two samples from Wilkinson County, GA, USA. To address the possibility of having multiple genetic profiles in a single sample, festoons of Spanish moss were sifted through and two single strands from the same festoon were separated for further analysis.

Microsatellite selection and amplification

We evaluated several different thermal cycling conditions to reduce nonspecific product. For the first set of loci (*VgC01*, *VgF02*, *Ai4.10*, *AC11*, *VS10*, *Dd03*, *e6*, *e19* and *e6b*), we used the touchdown PCR protocol described in Palma-Silva et al. (2007). This protocol requires that the denaturation and extension phases remain constant at 94°C and 72°C for 30 s each, but the annealing temperature (T_a) is decreased one degree at every step from 58°C to 48°C for 30 s with the last step of a 48°C T_a, repeated for 25 cycles. The other touchdown PCR protocol described by Wöhrmann and Weising (2011) was used on loci *Acom82.8*, *Acom9.9* and *Acom119.1*. The T_a started at 65°C and was decreased by one degree until it reached 54°C and was then repeated for 19 cycles.

For loci *Dd20*, *PaD07* and *p2p19*, touchdown PCR proved unsuccessful because there was no product found when size fractionated gel electrophoresis. It was found that amplification could be improved by using a gradient PCR protocol to determine what the

optimal annealing temperature was for that primer set and then running those samples separately (Table 6). The PCR conditions for these three primers were: initial denaturation of 95°C for 3 mins, 95°C for 30 s, 48°C to 50°C for 30 s, 72°C for 1 min. This was repeated for 34 cycles, followed by a final extension of 72°C for 5 mins. All samples were run on a BioRad T100™ Thermal Cycler. To conserve reagents, PCR reaction volume was eventually reduced from 25 µl to 10 µl reactions: 5 µl of Promega GoTaq G2 Hot Start Colorless Master Mix, 2.2 µl of H_2O, 1.2 µl of DNA template, and 1.6 µl of forward and reverse primer.

Fragment analysis

Functional primers were bound to one of two dye labels, 6-FAM or HEX (Table 6). The same general PCR reaction was followed, but this time the labeled forward primer was incorporated in a 1:3 ratio with the unlabeled forward primer. Depending on the intensity at which the samples fluoresced, which was measured in relative fluorescence units, the final PCR product was diluted 4x–5x as to make sure the fluorescence signal was within the measurable range of the analyzer. To prepare for analysis, one microliter of PCR product was then added to 10 µl of diluted ladder, which consisted of 10 µl of ROX size standard and 1000 µl of HI-DI Formamide (Thermo Fisher Scientific, Woolston, Warrington, UK). Final PCR products were then packaged and sent to the DNA Sequencing Facility at Florida State University for genotyping using an Applied Biosystems 3730 Genetic Analyzer with capillary electrophoresis. Microsatellite amplicons were scored using Peak Scanner™ 2 software.

Genetic analysis

GenAlEx v.6.5 (Peakall & Smouse, 2006; Peakall & Smouse, 2012) was used to determine the number of alleles (*A*), observed heterozygosity (H_O), and expected heterozygosity (H_E, unbiased; Nei, 1973). GenAlEx v.6.5 was also used to test for deviations from Hardy-Weinberg equilibrium and to calculate Wright's *F*-statistics based on heterozygosity (Nei, 1977). Genepop on the Web (Raymond & Rousset, 1995) was used to test for linkage disequilibrium (LD) between all pairs of loci.

Results

Genetic Variation and marker identification

Of the 15 loci that were screened, seven were polymorphic (*AC11*, *Acom9.9*, *e6*, *e19*, *p2p19*, *VgF02* and *VS10*). The number of alleles per locus ranged from 2 to 12 alleles with a mean of seven alleles per locus (Table 8). The remaining eight loci (*Acom82.8*, *Acom119.1*, *Ai4.10*, *Dd03*, *Dd20*, *e6b*, *Pad07* and *VgC01*) were completely monomorphic and were excluded from any further analysis (Table 6). Locus *Pad07* had two alleles but it was heterozygous across all samples that were screened; for this reason, it was also excluded from further analysis. For the seven loci that were polymorphic (and excluding *Pad07*), when all genotypes were pooled into a single sample, H_O ranged from 0.026 to 0.667 (mean =0.394) while H_E ranged from 0.025 to 0.625 (mean = 0.439). F_{IS} and F_{IT} ranged from -0.838 to 0.262 (mean = -0.028) and from 0.080 to 0.955 (mean = 0.564), respectively (Table 8). Significant genotypic linkage disequilibrium was detected between three pairs of loci (*Ac11* and *VgF02*; *e19* and *Vs10*; *VgF02* and *Vs10*).

In Lowndes County alone, the number of alleles ranged from 2 to 10 with a total of a mean of 6.1 alleles per locus. Significant departure from Hardy-Weinberg was

detected for all but two loci, *Acom9.9* and *p2p19*. The other five were found to differ significantly from Hardy-Weinberg at either the level $P < 0.01$ or $P < 0.001$ (Table 8). Observed heterozygosity ranged from 0.077 to 1 (mean = 0.491) while expected heterozygosity ranged from 0.075 to 0.889 (mean = 0.683). The system of mating inbreeding coefficient, F_{IS}, ranged from -0.701 to 0.786 (mean = 0.179) (Table 8).

For seven of the eight samples used for this experiment, the genotypes for each pair of strands of *T. usneoides* were the same. However, this was not the case for one sample from Great Swamp in South Carolina for two loci (*Ac11* and *VgF02*). In *Ac11*, one strand of *T. usneoides* from the same festoon had a heterozygous genotype of 200/212 while the other stand was 202/212. For *VgF02*, the base pair difference was much greater; one strand was heterozygous, 178/192, while the other was homozygous, 194/194. Due to the limited number of samples collected from Wilkinson County, GA and Beaufort County, SC not all population genetic measures and tests were conducted for these sites. However, it should be noted that there were six private alleles (alleles found in only one population) found across four loci in Beaufort County, SC (Table 9).

Discussion

Using cross-species transference of published microsatellite DNA primer sets for the family Bromeliaceae, seven polymorphic loci were identified that were potentially useful for population genetic studies of *T. usneoides*. This could however be reduced even further to only five polymorphic loci, due to linkage disequilibrium found between *VgF02* and *Vs10*. Approximately 85% of the samples used in this study were from various locations around Lowndes County and these samples account for ~ 87% of the alleles found. All alleles found in the two samples from Wilkinson County, GA were

present in the Lowndes County, GA samples and vice versa. Beaufort County had six alleles that were not present in Lowndes County and Lowndes County had 17 alleles absent in Beaufort County. These findings might suggest that with geographic distance, individuals of *T. usneoides* become more genetically dissimilar, but more intensive sampling is required given that the number of samples collected from each location were limited in number.

Three of the seven polymorphic primer sets (*e6*, *e19*, *p2p19*) were derived from a member of the same genus as our study organism, *Tillandsia usneoides*. These three loci had the lowest polymorphism with a fourth locus being completely monomorphic (*e6b*). Other loci from completely different genera such as *VgF02* (*Vriesea gigantea*), *Vs10* (*Vriesea simplex*) and *AC11* (*Aechmea caudata*) exhibited much greater polymorphism within our study organism. Previous studies demonstrated that *VgF02*, *Vs10* and *AC11* consistently showed amplification across numerous genera within the subfamily Tillandsioidae (Goetze et al. 2013; Neri et al., 2015; Palma-Silva et al., 2007). It is not uncommon for researchers to obtain functional markers through cross-species transference, especially in bromeliads. In fact, they are considered an exception in plants with transfer rates of approximately 10% between genera (Goetze et al., 2013). This may be because of the recent adaptive radiation of the family which would cause low sequence divergence (Goetze et al., 2013; Neri et al., 2015).

Because of limited sample sizes at each sampling location, estimates of population genetic parameters should be viewed cautiously Observed and expected heterozygosity differed significantly across five of the seven loci when samples were pooled across all sampling locations (Table 8). F_{IS} values were negative for loci *Acom9.9*,

e6 and *p2p19* meaning these loci showed an excess of heterozygosity, whereas the other four exhibited a heterozygote deficiency. In assessing the Lowndes County samples alone, it was found that five of the seven loci deviated significantly from Hardy-Weinberg expectations (Table 8). High F_{IS} values for loci *AC11*, *e19*, *VgF02* and *Vs10* indicated there was a heterozygote deficiency, which could be the result of inbreeding, null alleles, or limited sample sizes. Despite this, our results were found to be similar with other studies performed on epiphytic organisms in that they had similar values of population genetic parameters such as F_{IS}, F_{IT}, H_O and H_E (Avila-Diaz & Oyama, 2007; Cascante-Marin et al., 2014; Cortes-Palomec et al., 2019; Lavor et al., 2014; Trapnell et al., 2004; Zanella et al., 2011).

Other studies performed on species of Bromeliaceae that have shown similar F_{IS} and F_{IT} values and large disparities between H_O and H_E have attributed this pattern to an autogamous mating strategy (Lavor et al. 2014, Cascante-Marin et al. 2014). Lavor et al. (2014) and Zanella et al. (2011) both suggest that such patterns are not uncommon in the family Bromeliaceae. The Tillandsioideae subfamily has been shown to have high frequency of autogamy and mixed systems in other genera such as *Alcantarea*, *Guzmania*, *Racinea*, *Tillandsia*, *Vriesea* and *Werauhia* (Zanella et al., 2012 a). *Tillandsia usneoides* has the ability to vegetatively reproduce, which is similar to the asexual process of cloning which is seen in other species of Bromeliaceae. It has been found that cloning can increase generation time and promote the overlap of generations resulting in a reduction of the effects of genetic drift (Goetze et al., 2015). This was found to be the case in the bromeliad, *Aechmea tuitensis*, there they found that its clonal behavior preserved genotypes (Izquierdo & Piñero, 2000). However, for *T. usneoides* we found

significant heterozygote deficiencies across some loci which could indicate that the effects of genetic drift might be more prominent in our study species. Studies on marker development for the family Bromeliaceae have shown that when loci deviate from Hardy-Weinberg and have heterozygote deficiency such as the loci in our study, it is usually attributed to processes such as inbreeding, Wahlund effects and null alleles (Goetze et al., 2013; Neri et al., 2015; Paggi et al., 2008; Zanella et al., 2012a). Unfortunately, larger sample sizes within each locale would be required to determine whether such deficiencies are the result of similar processes or merely a result of sampling error.

Conclusion

We have found that out of 36 primer sets obtained from various taxa, only seven proved useful. Our success rate (~20%) was consistent, if not higher, than that reported for other studies of cross-species transference in the Bromeliaceae, but was disappointing considering that we targeted primer sets that were known to be conservative and/or that had produced visible fragments for *T. usneoides* in the cross species transference table. Of the 36 primer sets that were originally screened, we narrowed the set down further to 15 loci that produced fragments that were close in size to what was reported in the primer notes, but only 7 of the 15 loci produced polymorphic loci without other apparent abnormalities. Because our sample sizes at each location were limited, we cannot yet rule out other potential molecular genetic problems, such as null alleles, that might further limit the utility of the seven primer sets that we have identified. Although limited in number, the primer sets that we identified should still be sufficient for assessing population genetic patterns in *T. usneoides* and could also be used to examine fine-scale spatial genetic structure if sample sizes are sufficiently large (Banks & Peakall, 2012;

Binns et al., 2020) While it is not impossible that more primer sets could be developed, moving forward, it might be more time efficient to try SNPs, since high-throughput methods such as this make it possible to develop hundreds of markers in a short amount of time.

In addition to the challenge of identifying useful genetic markers for *T. usneoides*, this study has identified other potential challenges to working with this species. First, it can be difficult to determine how you should sample Spanish moss from a tree that has complete coverage of the organism versus one that is scantily covered. Because *T. usneoides* is distributed in three dimensions, a possible first step would be to study the fine-scale genetic structure of the organism within a tree (Trapnell et al., 2004); this would help resolve the potential differences in genetic structure between horizontal and vertical plans within a host tree. Not only is there a question of how *T. usneoides* should be sampled from a host tree, but also within a festoon itself. As our results show, some festoons of Spanish moss contain multiple genets. Billings (1904) reported that Spanish moss is more likely to be propagated by fragments of festoons than by seeds, however Garth (1964) dissected 30 flower buds and found that 26 had pollen on the stigma indicating that mechanical dispersion of fragments is not the only way for *T. usneoides* to spread throughout the environment. Naturally, it can be assumed that during dispersion Spanish moss strands can potentially intermingle to form festoons that consist of several different genets, or that festoons formed from different genets may intermingle to the point that they are indiscernible. Because the scope of our sampling was limited, further work is required to determine the frequency with which different genets might mix within

a festoon. Nevertheless, this study suggests that samples used for DNA extraction should be restricted to a single strand.

Additional challenges include the range from which samples are taken. Because the range extends (sometimes continuously) from the Andes of South America to the southeastern United States, a proper population genetic study will require extensive sampling over a wide area, with a sufficiently large number of samples per location to characterize the degree of population genetic variation and structure in this species. Because of the hierarchical nature of epiphytes such as *T. usneoides*, AMOVA might be an approach to consider, as it can assess genetic differentiation at varying levels, including festoon and tree. These changes would allow for a more extensive population genetic study on *T. usneoides* to be conducted; wherein linkage disequilibrium and population genetic parameters can be properly assessed. We would then be able to determine how *T. usneoides* is distributed and make inferences on its different reproductive modes.

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APPENDIX A:
Figures

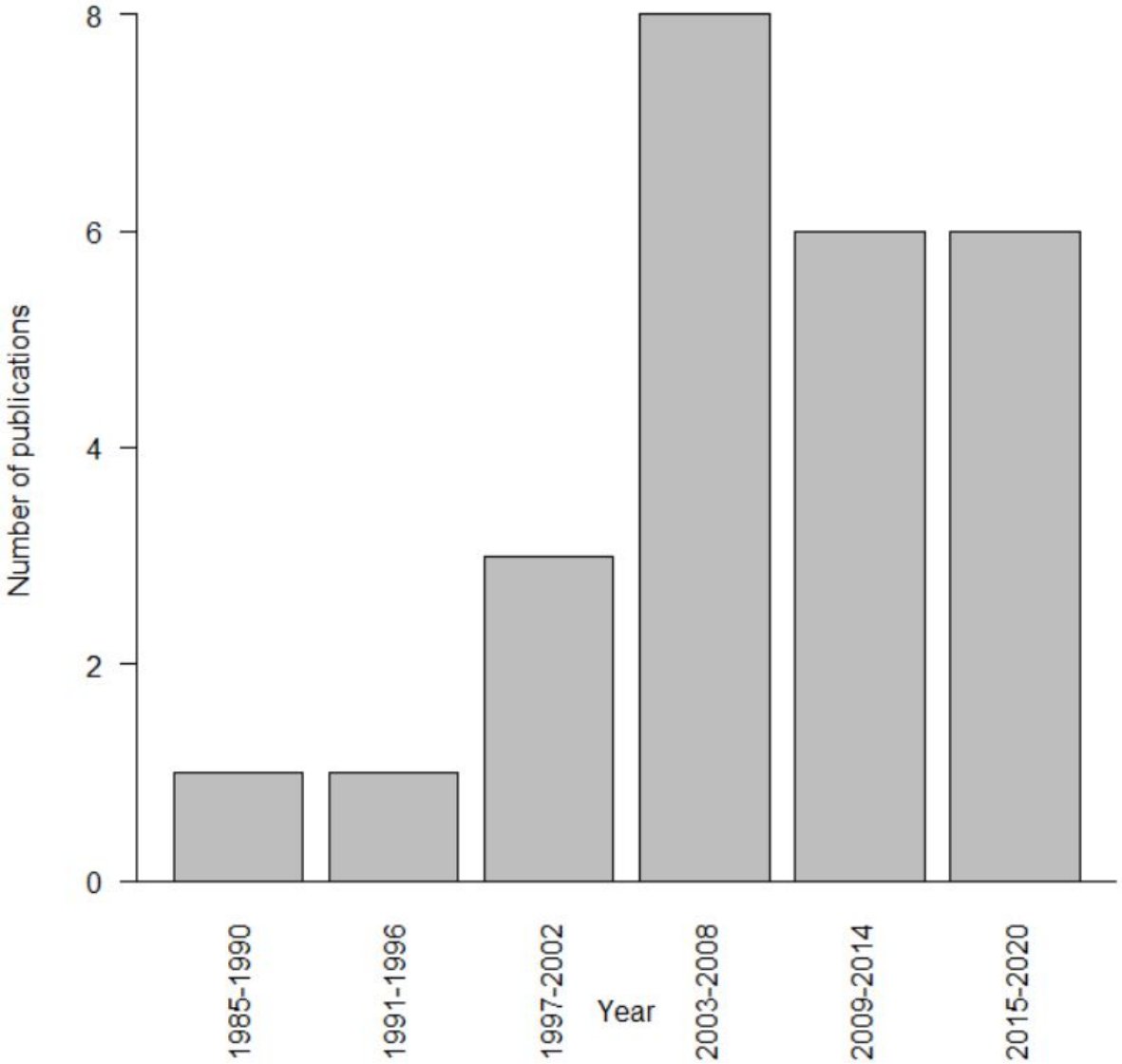

Figure 1. The number of publications on population genetics of epiphytes from 1985 to the current date. Since the first published paper in 1987, there have been 25 articles. In the five-year span from 2003 to 2008, the number of publications more than doubled from the previous five-year span but has since declined.

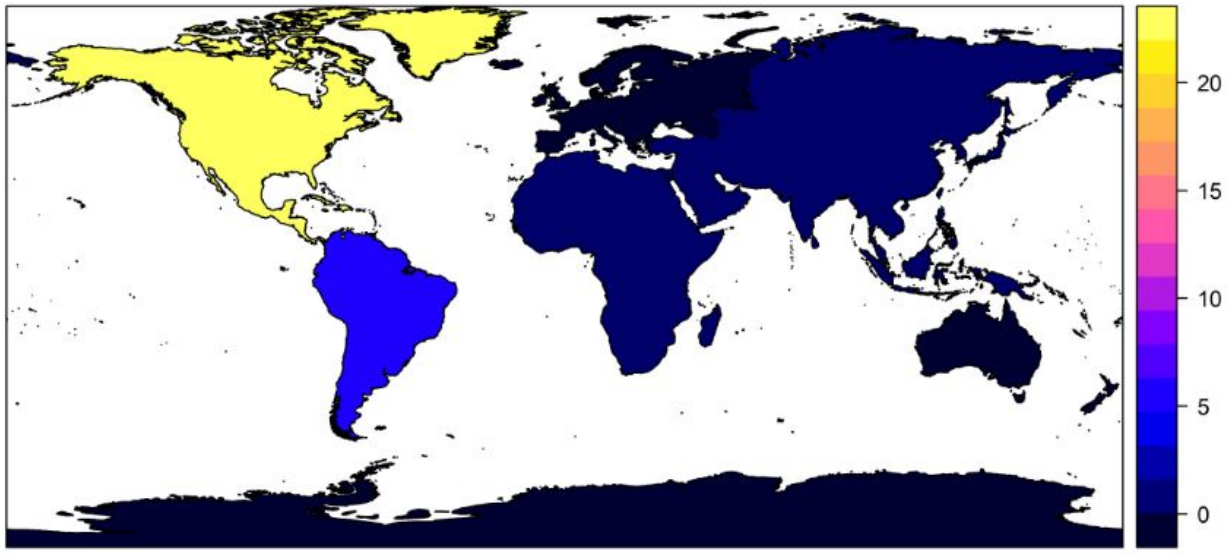

Figure 2. Number of population genetic studies of epiphytes per continent. The color gradient represents where the samples were collected, not necessarily where the genetic work was done. Black represents zero samples and yellow represents 20 or more. Species were mostly collected in the Neotropics (i.e. Mexico and Costa Rica) and the United States.

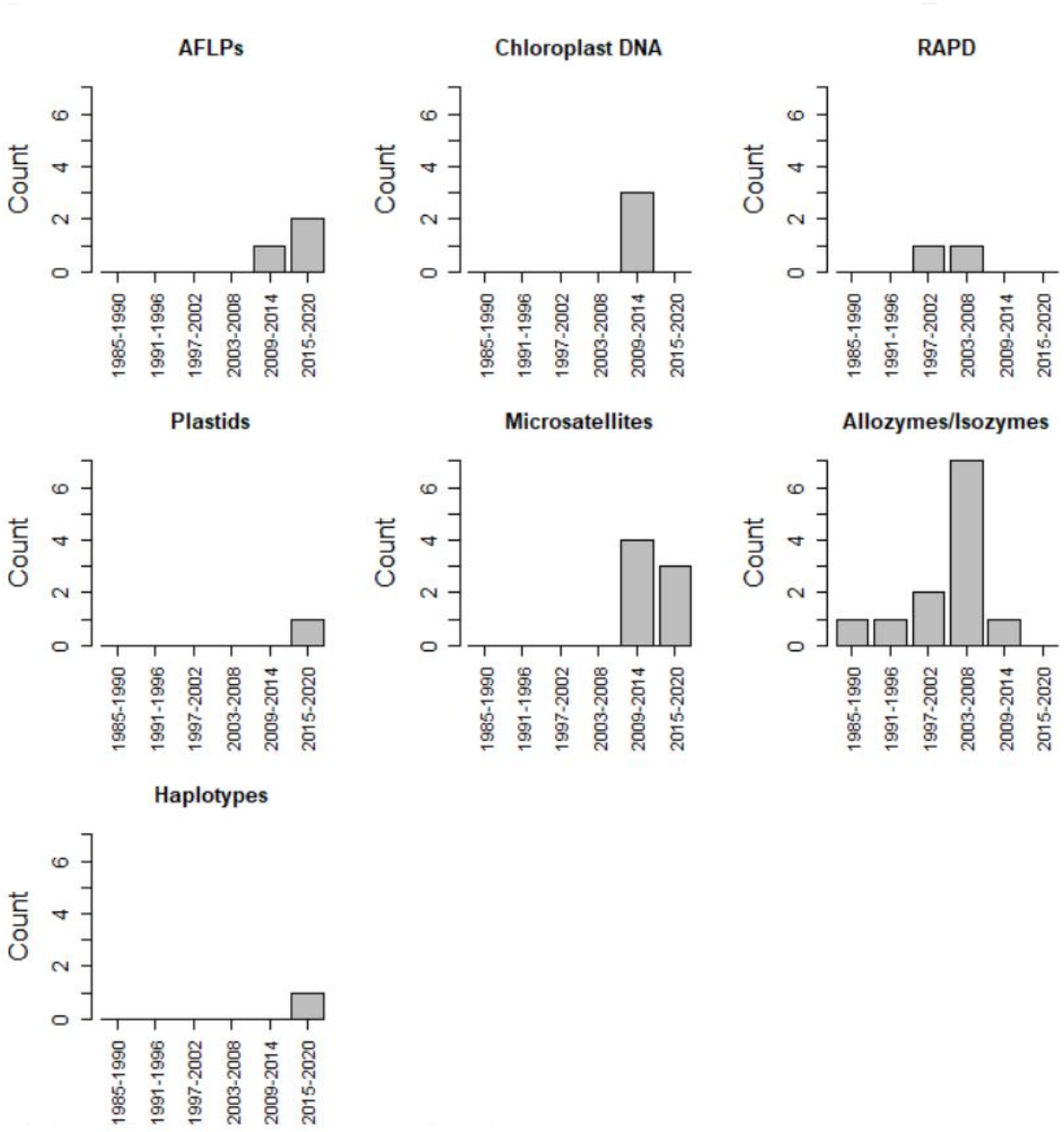

Figure 3. Trends in molecular marker usage in published population genetic studies of epiphytes. Seven different markers were used across 25 published papers. These seven makers were used 28 eight times overall. Allozymes/isozymes have been used most consistently through time.

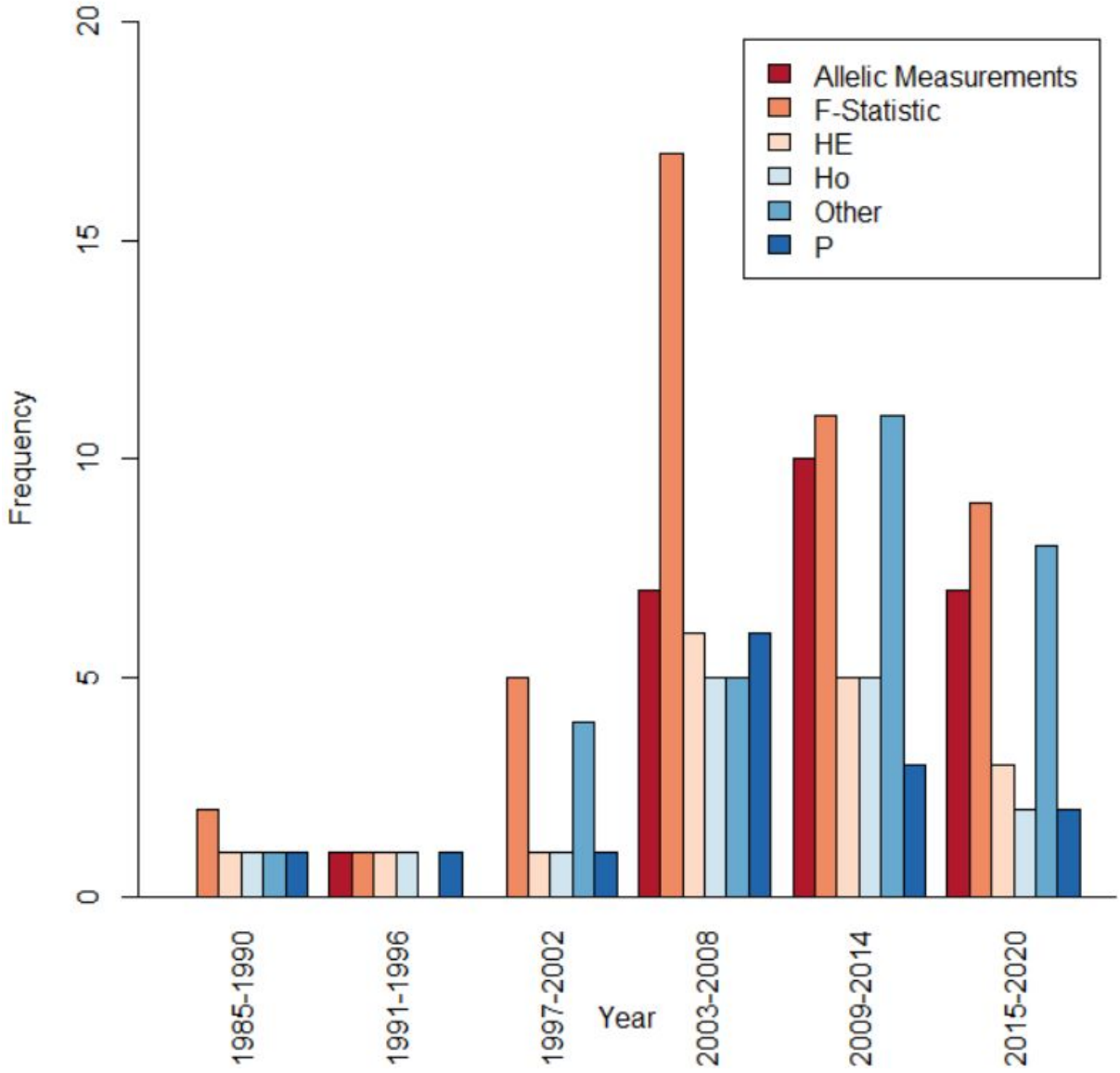

Figure 4. The five most commonly used population genetic measures are displayed, measures used less frequently are grouped in the "other" category. Expected heterozygosity (H_E), observed heterozygosity (H_O), proportion of polymorphic loci (P).

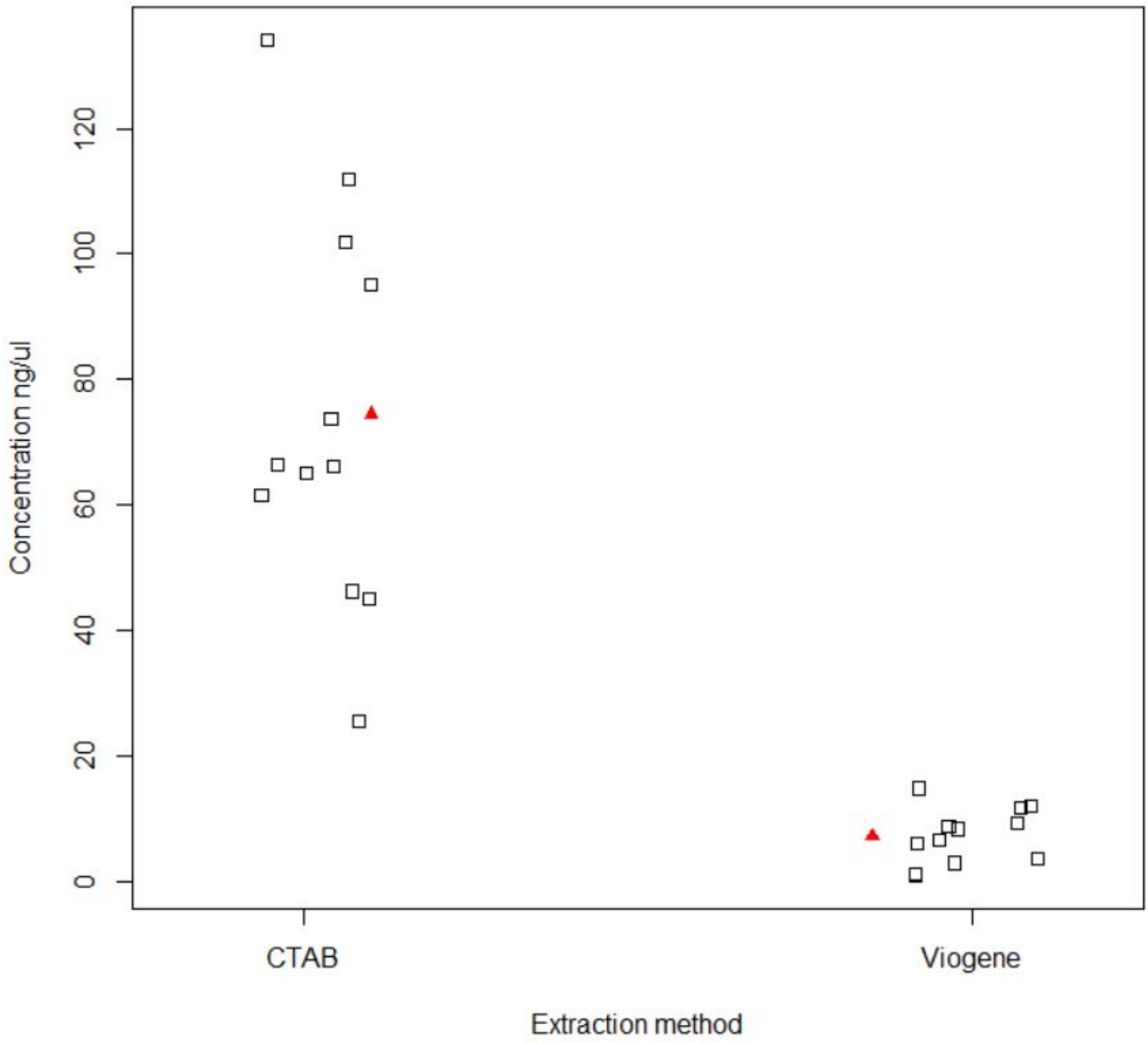

Figure 5. Comparison of a commercial DNA extraction kit (Viogene) to a modified CTAB extraction method. CTAB resulted in significantly higher yields of DNA than Viogene, albeit with a larger standard deviation. Average DNA yields for each method are represented by red triangles.

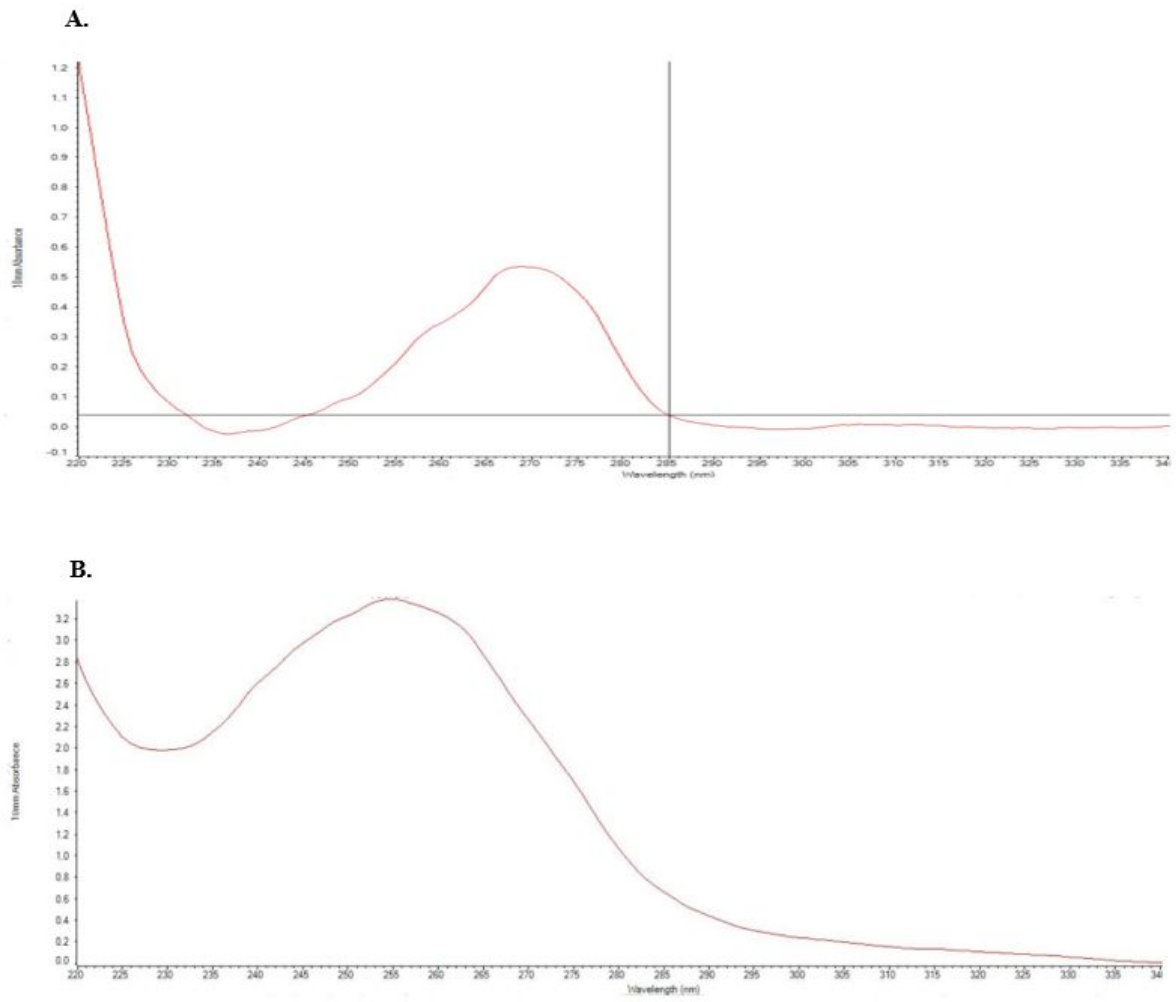

Figure 6. Spectra produced from the Nanodrop that shows the difference in phenol contamination before (**A**) adding the extra chloroform clean up step and after (**B**).

APPENDIX B:
Tables

Table. 1 Frequency of population genetic studies of epiphytes by country and continent. The number of species studied in each taxon by country is also displayed. Here, Polypodiaceae is equal to Orchidaceae due to the fact there are studies that focus on several different species in a survey.

Country	Number of studies in Country	Continent	Number of studies on Continent	Number of species studied per taxon/country		
				Bromeliaceae	Orchidaceae	Polypodiaceae
Brazil	4	Africa	1	3	1	-
Costa Rica	8	Asia	1	2	5	3
Ecuador	1	North America	20	-	1	-
Madagascar	1	South America	5	-	1	-
Mexico	7	-	-	4	2	5
South Korea	1	-	-	-	2	-
United States	5	-	-	1	5	8

Table 2. These are population genetic measures that fall into the "other" category. They were seldom used across the 25 population genetic studies of vascular epiphytes.

Population genetic measure	Description
I	Nei's unbiased genetic identities
N_m	Number of migrants per generation
H_w	Average gene diversity within populations
H_b	Average diversity among populations
H	Number of haplotypes
PH	Private haplotypes
HD	Haplotype diversity
S	Segregating sites
P_{ij}	Coancestry coefficient
P_i	Nucleotide diversity

Table. 3 All journals that published one or more of the peer-reviewed articles on population genetics of epiphytes (included in this review).

Journal	Founding year	Number of publications	Impact Factor	
			Most recent	5 - year
Conservation Genetics	2000	1	2.283	2.188
Genetics and Molecular Biology	1998	1	2.127	2.005
Biodiversity and Conservation	1992	1	3.142	3.295
Molecular Ecology	1992	5	5.855	-
Selbyana	1975	3	-	-
Biological Journal of the Linnean Society	1969	1	2.203	2.243
Biotropica	1969	2	2.989	-
Journal of the Bromeliad Society	1950	1	-	-
Heredity	1947	3	3.179	3.677
American Journal of Botany	1914	3	2.8	3.407
Annals of Botany	1887	2	3.454	-
Botanical Journal of the Linnean Society	1858	1	3.051	2.808

Table. 4 The seven publications in which the microsatellite primers used in this study were obtained.

Publication	Source taxon	Subfamilies tested	Number of species tested	Amplification in Tillandsia usneoides
Boneh et al. (2003)	*Tillandsia fasciculata* *Guzmania monostachya*	-	2	Not tested
Goetze et al. (2013)	*Aechmea caudata*	Bromelioideae Pitcairnioideae Tillandsioideae	21	Not tested
Neri et al. (2015)	*Vriesea simplex*	Bromelioideae Pitcairnioideae Tillandsioideae	10	Not tested
Paggi et al. (2008)	*Pitcairnia albiflos*	Bromelioideae Pitcairnioideae Tillandsioideae	16	Not tested
Palma-Silva et al. (2007)	*Vriesea gigantea* *Alcantarea imperialis*	Bromelioideae Pitcairnioideae Tillandsioideae	22	Not tested
Wöhrmann and Weising (2011)	*Ananas comosus*	Bromelioideae Pitcairnioideae Tillandsioideae	74	+
Zanella et al. (2012)	*Dyckia distachya*	Bromelioideae Pitcairnioideae Tillandsioideae	22	Not tested

Table. 5 Geographic coordinates and sample sizes for locations where *Tillandsia usneoides* was collected in Lowndes (A), Wilkinson (B) and Beaufort (C) counties. Lowndes County alone accounts for about 85% of the samples collected. Samples per site indicates the number of samples used in the analysis, not how many were collected (some samples were not used or produced insufficient yields of genomic DNA).

Sampling Locations		
Lowndes county, GA	Samples per site	Longitude/Latitude
Clyattville	2	30°41’32.9” N, 83°18’54.7” W
Hahira	3	30°59’19.9” N, 83°21’59.4” W
Naylor	2	30°54’45.5” N, 83°04’13.0” W
Remerton	1	30°50’42.0” N, 83°18’21.9” W
1411 New Statenville Hwy	5	30°48’45.3” N, 83°14’11.1” W
Freedom Park	1	30°54’38.2” N, 83°14’52.6” W
4990 Hammock Trail	3	30°41’16.9” N, 83°13’10.7” W
Lake Sheri	2	30°51’03.6” N, 83°19’47.6” W
Oris Black Burn memorial park	1	30°39’58.0” N, 83°19’12.0” W
Wiregrass community college	2	30°53’23.0” N, 83°21’12.3” W
4511 Briggston Road	2	30°43’20.3” N, 83°17’55.0” W
3336 Brown Road	3	30°44’32.0” N, 83°15’48.2” W
Lake Louise	2	30°43’32.0” N, 83°15’21.8” W
Valdosta State University	1	30°50’55.4” N, 83°17’15.9” W
Wilkinson County, GA		
Hardie Cemetery	1	32°54’47.6” N, 83°19’47.3” W
Gordon City Cemetery	1	32°52’45.3” N, 83°20’01.5” W
Beaufort County, SC		
101 Marshland Road	1	32°11’55.6” N, 80°43’08.3” W
Great Swamp	1	32°15’06.9” N, 81°00’31.4” W
9 Amelia Circle	1	32°13’19.3” N, 80°45’29.8” W

Table 6. Description of all 15 loci used in the cross-species transference study for *Tillandsia usneoides*. Annealing temperature (T_a) represents the temperature 5°C lower than the lowest melting temperature in each primer set.

Locus	Dye label	Repeat motif	T_a	Source species	Publication
AC11*	6-FAM	$(CA)_X$	55°C	*Aechmea caudata*	Goetze et al. (2013)
Acom9.9*	Hex	$(TTC)_X$	47.8°C	*Ananas comosus*	Wöhrmann and Weising (2011)
Acom82.8	Hex	$(GT)_X$	47.9°C	*Ananas comosus*	Wöhrmann and Weising (2011)
Acom119.1	6-FAM	$(CTTT)_X$	48°C	*Ananas comosus*	Wöhrmann and Weising (2011)
Ai4.10	Hex	$(AT)_X(CATG)_X$	48.3°C	*Alcantarea imperialis*	Palma-Silva et al. (2007)
Dd03	6-FAM	$(CA)_X$	48.3°C	*Dyckia distachya*	Zanella et al. (2012)
Dd20	6-FAM	$(CA)_X$	50.1°C	*Dyckia distachya*	Zanella et al. (2012)
e6*	Hex	$(CAA)_X$	46°C	*Tillandsia fasciulata*	Boneh et al. (2003)
e6b	Hex	$(CAA)_X$	47°C	*Tillandsia fasciulata*	Boneh et al. (2003)
e19*	Hex	$(CT)_X$	45.9°C	*Tillandsia fasciulata*	Boneh et al. (2003)
p2p19*	6-FAM	$(GAA)_X$	48.2°C	*Tillandsia fasciulata*	Boneh et al. (2003)
PaD07	6-FAM	$(TG)_X$	49.6°C	*Pitcairnia albiflos*	Paggi et al. (2008)
VgC01	6-FAM	$(CT)_X$	50°C	*Vriesea gigantea*	Palma-Silva et al. (2007)
VgF02*	6-FAM	$(CT)_X(CT)_X$	47.7°C	*Vriesea gigantea*	Palma-Silva et al. (2007)
Vs10*	Hex	$(AC)_X(CT)_X$	48.2°C	*Vriesea simplex*	Neri et al. (2015)

* Indicates loci that were polymorphic and used for genetic analysis.

Table 7. The 36 original primer sets. These were obtained all from members of the family Bromeliaceae, developed from 10 different species.

Locus	Source Species	Publication
AC11	*Aechmea caudata*	Goetze et al. (2013)
AC25	*Aechmea caudata*	Goetze et al. (2013)
Acom9.9*	*Ananas comosus*	Wöhrmann and Weising (2011)
Acom12.12*	*Ananas comosus*	Wöhrmann and Weising (2011)
Acom78.4*	*Ananas comosus*	Wöhrmann and Weising (2011)
Acom82.8*	*Ananas comosus*	Wöhrmann and Weising (2011)
Acom117.5*	*Ananas comosus*	Wöhrmann and Weising (2011)
Acom119.1*	*Ananas comosus*	Wöhrmann and Weising (2011)
Ai4.10**	*Alcantarea imperialis*	Palma-Silva et al. (2007)
CT5	*Guzmania monostachya*	Boneh et al. (2003)
Dd03	*Dyckia distachya*	Zanella et al. (2012)
Dd10	*Dyckia distachya*	Zanella et al. (2012)
Dd20	*Dyckia distachya*	Zanella et al. (2012)
e6**	*Tillandsia fasciulata*	Boneh et al. (2003)
e19**	*Tillandsia fasciulata*	Boneh et al. (2003)
e6b**	*Tillandsia fasciulata*	Boneh et al. (2003)
Op13	*Orthopytum ophiuroides*	Aoki-Concalves et al. (2014)
Op77	*Orthopytum ophiuroides*	Aoki-Concalves et al. (2014)
Op89	*Orthopytum ophiuroides*	Aoki-Concalves et al. (2014)
P2p19**	*Tillandsia fasciulata*	Boneh et al. (2003)
PaA10**	*Pitcairnia albiflos*	Paggi et al. (2008)
PaC05**	*Pitcairnia albiflos*	Paggi et al. (2008)
PaD07**	*Pitcairnia albiflos*	Paggi et al. (2008)
PaZ01**	*Pitcairnia albiflos*	Paggi et al. (2008)
VgA04**	*Vriesea gigantea*	Palma-Silva et al. (2007)
VgB06**	*Vriesea gigantea*	Palma-Silva et al. (2007)
VgB10**	*Vriesea gigantea*	Palma-Silva et al. (2007)
VgC01**	*Vriesea gigantea*	Palma-Silva et al. (2007)
VgF02**	*Vriesea gigantea*	Palma-Silva et al. (2007)
VgG05**	*Vriesea gigantea*	Palma-Silva et al. (2007)
Vs1	*Vriesea simplex*	Neri et al. (2015)
Vs8	*Vriesea simplex*	Neri et al. (2015)
Vs9	*Vriesea simplex*	Neri et al. (2015)
Vs10	*Vriesea simplex*	Neri et al. (2015)
Vs17	*Vriesea simplex*	Neri et al. (2015)
Vs20	*Vriesea simplex*	Neri et al. (2015)

*Amplified using *T. usneoides*, ** Amplified on species in genus *Tillandsia*

Table 8. Samples per locus (*N*), number of alleles (*A*), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}) and the overall fixation index (F_{IT}) for each of the seven polymorphic loci that were screened over the 16 sample populations for *Tillandsia usneoides*. Includes all populations and Lowndes County alone. Other loci were not included as they were completely monomorphic.

	Total						Lowndes			
Locus	N	A	H_O	H_E	F_{IS}	F_{IT}	A	H_O	H_E	F_{IS}
AC11***	34	9	0.391	0.588	0.262	0.582	7	0.174	0.830	0.786
Acom9.9	34	7	0.549	0.625	-0.013	0.080	6	0.813	0.774	-0.083
e6***	34	4	0.667	0.422	-0.838	0.630	4	1	0.6	-0.701
e19***	34	6	0.397	0.516	0.147	0.467	5	0.190	0.747	0.739
VgF02**	26	12	0.513	0.608	0.058	0.457	10	0.538	0.889	0.370
Vs10**	26	9	0.216	0.288	0.227	0.780	9	0.647	0.863	0.227
p2p19	34	2	0.026	0.025	-0.040	0.955	2	0.077	0.075	-0.040

** $P < 0.01$, ***$P < 0.001$; indicates loci that deviated from HWE.

Table 9. The private and shared alleles found in Lowndes and Beaufort County. Four of the seven polymorphic loci had private alleles that were only found in distant populations.

Locus	Lowndes County	Beaufort County	Shared
AC11	196, 202, 206, 210	200, 212	204, 208, 214
Acom9.9	136, 138, 140, 143	146	130,133
e19	110, 134	108	112, 124, 130
VgF02	170,174,182, 184, 194, 198, 204	176, 178	180, 190, 192